Vietnam Journal of Science and Technology ***55*** *(5A) (2017) 66-73*

INTERACTION EFFECTS OF *LITSEA CUBEBA* ESSENTIAL OIL AND ANTIBIOTICS ON ANTIBACTERIAL ACTIVITY AGAINST PATHOGENIC BACTERIA IN AQUACULTURE

Hai Van Nguyen[1, 2], Thu Trang Vu[1], Son Chu-Ky[1], Samira Sarter[2, *]

[1]*School of Biotechnology and Food Technology, Hanoi University of Science and Technology, 1 Dai Co Viet, Hai Ba Trung, Ha Noi 10000, Viet Nam*

CIRAD, UMR ISEM, Montpellier F-34398, France

[*]Email: *samira.sarter@cirad.fr*

Received: 15 August 2017; Accepted for publication: 9 October 2017

ABSTRACT

In this study, the interaction effects of *Litsea cubeba* essential oil (EO) and two antibiotics (ABs) on the antibacterial activity against pathogenic bacteria in aquaculture were investigated. Two reference strains (*Escherichia coli* ATCC 25922 (*E. coli* ATCC 25922) and *Vibrio parahaemolyticus* ATCC 17802 (*V. parahaemolyticus* ATCC 17802)) and six locally isolated aquatic pathogenic strains (*E. coli* 9C48, *E. coli* 11C123, *Vibrio* 2S4, *Vibrio* 2N38, *V. parahaemolyticus* ND201 and *V. parahaemolyticus* TB81) were used. The inhibitory effects of individual antimicrobial agent (*L. cubeba* EO, nalidixic acid, and oxytetracycline) were tested against eight strains by using broth microdilution assay in 96-well microplates. A higher inhibitory effect of *L. cubeba* EO was observed against isolated aquatic pathogenic (MIC = 1.15 - 2.30 mg/mL) than that in the reference strains (MIC = 5.53 mg/mL). The combination effects of *L. cubeba* EO and ABs often used in the treatment of bacteria effects in aquaculture (nalidixic acid and oxytetracycline) were evaluated by the checkerboard method. Fractional Inhibitory Concentration (FIC) values were determined to characterize the interaction among combinations. Out of 16 AB-EO combinations tested, 11 of them showed a synergistic effect (FIC $\leq$ 0.5), 3 of them showed an additional effect (0.5 < FIC $\leq$ 1.0) and 2 of them showed indifferent effect (1 < FIC $\leq$ 4), no antagonistic effect was observed. The antimicrobial synergy of EO and AB could enhance efficacy, reduce toxicity, decrease adverse side effects, and lower the dose of ABs used in aquaculture.

Keywords: Litsea cubeba, essential oil, antibacterial activity, synergistic effect.

1. INTRODUCTION

According to the worldwide extension of aquaculture activity, new emerged diseases and the occurrence of other diseases increased year by year, in which, bacteria are a major cause of diseases [1]. *Vibrio* spp. and *E. coli* are the pathogenic bacteria which may be found in aquatic animals [2]. Since antibiotics (ABs) have the capacity to kill or inhibit the growth of bacteria by

having specific mechanisms toward the pathogens targeted, producers have to make use of massive amounts of ABs in order to control mortality and avoid huge economic losses. However, the use of a wide variety of ABs (e.g., oxytetracycline, nalidixic acid, amoxicillin, sulfonamides) [3] in large amounts, including non-biodegradable ABs useful in human medicine, ensures that they remain in the aquatic environment, exerting their selective pressure for long periods of time. This process has resulted in the emergence of AB-resistant bacteria in aquaculture environments, in the increase of AB resistance in fish pathogens, in the transfer of these resistance determinants to bacteria of land animals and to human pathogens, and in alterations of the bacterial flora both in sediments and in the water column. The use of large amounts of ABs that have to be mixed with fish food also creates problems for industrial health and increases the opportunities for the presence of residual ABs in fish meat and fish products [4]. Thus the reduction in ABs consumption in aquaculture is clearly important to minimize this problem. Plant secondary metabolites, e.g. essential oils (EOs) are known to display antimicrobial properties that could be relevant alternatives to ABs.

EOs are products of the secondary metabolites formed by aromatic plants. May Chang *Litsea cubeba* (so-called "Màng tang" in Vietnamese), a plant of *Litsea* genera that has been known for a long time as a popular remedy, is a potential therapeutic plant. *L. cubeba* has been used in traditional medicine to treat headache, fatigue, muscle pain, depression, sores and furuncles [5]. Its extracts have been reported for their antibacterial, antifungal, antioxidant and anticancer activities [5]. However, in many case, natural antimicrobial compounds from plant possess relatively weak activity, compared to synthetic ABs.

The combination of antibacterial agents is based on the Principe that the formulation may enhance efficacy, reduce toxicity, decrease adverse negative effects, lower dose of antibacterial agents [6]. Various antimicrobial interactions have been reported for EOs and ABs when tested in binary combinations [7 - 10]. For instance, lemongrass (*Cymbopogon citratus*) EO and its main component, citral, combined with streptomycin and kanamycin exhibited synergistic or additive effect against *Salmonella typhimurium* [9]. Oregano (*Origanum vulgare*) EO in combination with doxycycline, florfenicol or sarafloxacin possessed synergistic effects against *E. coli* isolated from chickens [10]. However, to the best of our knowledge, the combination of antibacterial activities between *L. cubeba* EO and ABs against reference and isolate strains for aquaculture have not been reported yet.

The aim of this work was to investigate the antibacterial effects of *L. cubeba* EO and nalidixic acid and oxytetracycline individual and in combination against two reference strains (*E. coli* ATCC 25922, and *V. parahaemolyticus* ATCC 17802) and 6 isolated aquatic pathogenic strains (*E. coli* 9C48, *E. coli* 11C123, *V. parahaemolyticus* ND201, *V. parahaemolyticus* TB81, *Vibrio* 2S4 and *Vibrio* 2N38).

2. MATERIALS AND METHODS

2.1. Materials

L. cubeba EO purchased from Aromasia company (> 98 % pure) (Vietnam), and nalidixic acid and oxytetracycline (Sigma Aldrich, France) were tested towards eight strains including: two reference strains: *E. coli* ATCC 25922, *V. parahaemolyticus* ATCC 17802; two *Vibrio* spp. isolated strains *V. parahaemolyticus* ND201 and *V. parahaemolyticus* TB81 isolated from the whiteleg shrimp *Litopenaeus vannamei* Acute Hepatopancreatic Necrosis Syndrome (AHPNS) hatchery of local farming in Nam Dinh and Thai Binh (National Veterinary Diagnostic Center –

Hanoi); four isolated strains kindly provided by the Pathology Laboratory of the Department of Aquatic Animal Pathology, Faculty of Fisheries, Nong Lam University including *Vibrio* 2S4, *Vibrio* 2N38, and *E. coli* 9C48, *E. coli* 11C123 isolated from cockle and catfish, respectively.

2.2. Determination of minimum inhibition concentration (MIC)

The minimum inhibition concentration (MIC) of the antibacterial agents (EO and AB) was determined using microbroth dilution assay in 96-well microplates. The EO was dissolved in distillated water containing 0.5 % Tween 80 to achieve a final concentration ranging from 0.195 µL/mL to 50 µL/mL. Bacterial cultures were prepared in Mueller Hilton Broth (MHB, Merck) or MHB plus NaCl 2 % for vibrios. An overnight culture was prepared and adjusted to obtain a suspension of 10^7 CFU/mL by measuring the Optical Density (OD) at 600 nm. Each well contained 20 µL of test sample, 20 µL of bacterial suspension and 160 µL MHB. Thus, the final bacterial concentration for the assay was then 10^6 CFU/mL. After incubation for 24 h at 28 ^oC for vibrios and at 37 ^oC for *E. coli*, the OD was measured at 600 nm using Elisa reader (Bio-rad Model 680, Japan). The MIC was determined as the lowest concentration showing no growth [5]. The assays were carried out in triplicate. A positive control containing the bacterial culture without the EO and a negative control containing the MHB, Tween and EOs were performed in the same conditions [5].

2.3. Checkerboard method: interaction effect on antibacterial activity of *L. cubeba* and AB

The checkerboard method was performed using 96-well microplates to obtain the fractional inhibitory concentration (FIC) index [8]. Six serial (2, 1, 1/2, 1/4, 1/8 and 1/16 MIC), two-fold dilutions of *L. cubeba* EO (A) and antibiotics (B) were prepared as in the MIC tests. 10 µL of EO dilution were added to the plate in a vertical orientation and 10 µL of AB dilution were added in a horizontal orientation. Then, 20 µL of the suspension bacterial overnight culture containing 10^7 CFU/mL of the strain were added to all wells. 160 µL of media MHB were filled to obtain total of 200 µL. Plates were then incubated at 37 ^oC for *E. coli* and 28^oC for vibrios for 24 h.

The FIC index was calculated as $\sum FIC = FIC_A + FIC_B$

where:

$$FIC_A = \frac{MIC_A combination}{MIC_A alone};$$

$$FIC_B = \frac{MIC_B combination}{MIC_B alone}$$

The results were interpreted as synergistic (FIC $\leq$ 0.5), additional (0.5 < FIC $\leq$ 1), indifferent (1 < FIC $\leq$ 4) or antagonistic (FIC > 4) [8]. Experiments were performed in triplicate.

3. RESULTS AND DISCUSSION

The antimicrobial activity individual and in combination of EO and AB (MIC and FIC values) against *Vibrio* spp. and *E. coli* were presented in Table 1 and Table 2, respectively.

Table 1. FIC values and the combinations effects of *L. cubeba* fruit EO and antibiotic against *Vibrio* spp.

Strains	Antimicrobial agents	MIC (mg/mL)		FIC	Sum FIC	Interaction
		Alone	Combination			
V. parahaemolyticus ATCC 17802	*L. cubeba*	5.53	0.69	0.13	0.23	Synergy
	Nalidixic acid	$2.67.\ 10^{-3}$	$0.27.\ 10^{-3}$	0.10		
	L. cubeba	5.53	0.61	0.11	0.28	Synergy
	Oxytetracycline	$2.67.\ 10^{-3}$	$0.45.\ 10^{-3}$	0.17		
Vibrio 2S4	*L. cubeba*	1.15	0.77	0.67	1.17	Indifference
	Nalidixic acid	$64.0.\ 10^{-3}$	$32.0.\ 10^{-3}$	0.50		
	L. cubeba	1.15	0.96	0.83	1.33	Indifference
	Oxytetracycline	$16.0.\ 10^{-3}$	$8.0.\ 10^{-3}$	0.50		
Vibrio 2N38	*L. cubeba*	2.30	0.97	0.42	0.92	Addition
	Nalidixic acid	$53.3.\ 10^{-3}$	$26.7.\ 10^{-3}$	0.50		
	L. cubeba	2.30	0.76	0.33	0.83	Addition
	Oxytetracycline	$8.0.\ 10^{-3}$	$4.0.\ 10^{-3}$	0.50		
V. parahaemolyticus ND201	*L. cubeba*	1.67	0.22	0.13	0.30	Synergy
	Nalidixic acid	$8.0.\ 10^{-3}$	$1.4.\ 10^{-3}$	0.17		
	L. cubeba	1.67	0.32	0.19	0.44	Synergy
	Oxytetracycline	$16.7.\ 10^{-3}$	$4.2.\ 10^{-3}$	0.25		
V. parahaemolyticus TB81	*L. cubeba*	1.75	0.37	0.21	0.38	Synergy
	Nalidixic acid	$9.3.\ 10^{-3}$	$1.6.\ 10^{-3}$	0.17		
	L. cubeba	1.75	0.19	0.11	0.32	Synergy
	Oxytetracycline	$16.0.\ 10^{-3}$	$3.4.\ 10^{-3}$	0.21		

The MIC values of *L. cubeba* EO against all tested strain ranging from 1.15 - 5.53 mg/mL. The same antibacterial activities (MIC = 1.25 - 10 mg/mL) were reported for *L. cubeba* EO rich in citral (83.9 %) from India [11]. Recent observations by transmission electron microscopy showed holes and gaps in the cell wall of *E. coli* and that the bacteria were killed or destroyed within 2 h when treated with 0.125 % v/v of *L. cubeba* [12].

In addition, *L. cubeba* EO had higher inhibitory effect against isolated strains than reference strains. Indeed, the MIC values against isolated strains lower 2.4 - 4.8 times (corresponding to 1.15 - 2.30 mg/mL) compared to those (5.53 mg/mL) against references strains. Generally, a higher amount of AB was required when treating bacteria resistant to AB. However, the effect of EOs to isolated bacteria was variable. A higher MIC values (MIC = 0.015 - 0.062 μg/mL) of clove (*Syzygium aromaticum*) and citronella (*Cymbopogon nardus*) EO

against 32 strains including *Vibrio* spp., *Edwardsiella* spp., *Aeromonas* spp., *E. coli*, isolated from aquatic

Table 2. FIC values and the combinations effects of *L. cubeba* fruit EO and antibiotic against *E. coli.*

Strains	Antimicrobial agents	MIC (mg/mL)		FIC	Sum FIC	Interaction
		Alone	Combination			
E. coli ATCC 25922	*L. cubeba*	5.53	0.44	0.08	0.33	Synergy
	Nalidixic acid	$4.0.\ 10^{-3}$	$1.0.\ 10^{-3}$	0.25		
	L. cubeba	5.53	1.38	0.25	0.40	Synergy
	Oxytetracycline	$1.67.\ 10^{-3}$	$0.25.\ 10^{-3}$	0.15		
E. coli 9C48	*L. cubeba*	1.38	0.08	0.06	0.14	Synergy
	Nalidixic acid	$16.0.\ 10^{-3}$	$1.3.\ 10^{-3}$	0.08		
	L. cubeba	1.38	0.08	0.06	0.14	Synergy
	Oxytetracycline	$32.0.\ 10^{-3}$	$2.6.\ 10^{-3}$	0.08		
E. coli 11C123	*L. cubeba*	2.30	0.58	0.25	0.50	Synergy
	Nalidixic acid	$21.3.\ 10^{-3}$	$5.3.\ 10^{-3}$	0.25		
	L. cubeba	2.30	0.97	0.42	0.92	Addition
	Oxytetracycline	$21.3.\ 10^{-3}$	$10.7.\ 10^{-3}$	0.50		

animals than those against reference strains (MIC = 0.015 μg/mL) were reported [13]. Otherwise, the better inhibitory effects were observed when compared the MIC values of *Cinnamomum cassia*, *C. verum*, *Origanum compactum*, *Thymus capitatus* and *T. vulgaris thymoliferum* against *Listeria monocytogenes*, *Salmonella* Typhimurium and *E. coli* isolated strains from meat than references strains [14].

In order to reduce the consumption of ABs as well as economic losses due to the use large quantities of EOs, the *L. cubeba* fruit EO were used in combination with ABs. The FIC indices ranged from 0.23 - 1.17 and from 0.14 - 0.92 for the combined antimicrobial substances against *Vibrio* spp. and *E. coli*, respectively. Overall, no antagonistic effect was observed for any of the combination evaluated. A majority of combination provided a synergistic effect against tested microorganisms. In fact, out of 16 AB-EO pairs tested, 11 of them showed synergistic effect ($FIC \leq 0.5$), 3 of them showed addition effect ($0.5 < FIC \leq 1.0$) and 2 of them showed indifferent effect ($1 < FIC \leq 4$). The best combination was found for *L. cubeba* and nalidixic acid, oxytetracycline against *E. coli* 11C123, in which FIC values were 0.14 and showed a total synergistic effect (MIC reduce 12 - 17 fold). A significant 4 to 10 times and 4 to 13 times reduction in the MIC of the ABs and *L. cubeba*, were observed in rest combinations with FIC index ranging from 0.23 to 0.50, respectively.

Several studies have described various interaction effects for ABs and EOs. Tea tree (*M. alternifolia*) EO combinations with gentamicin or tobramycin had a synergistic effect against *E. coli* and *S. aureus* [9]. The author suggested that aminoglycosides ABs inhibit protein synthesis and tea tree EO damages the cytoplasmic membrane of bacteria; possibly this combination possessed multi-target synergy [9]. The decrease of MIC values when used in

combination could be explained by the presence of various antimicrobial components. Since many EOs or EO compounds generally affected the cell membranes of bacteria and most ABs have specific targets in protein or DNA synthesis, it seems likely that synergy in most cases may be due to multi-target effects. The multi-targets include receptor site modification, enzymatic degradation, reduced accumulation of drug within the bacterial cell, decreased membrane permeability, and efflux pumps [6]. In addition, some mechanisms pathways of EO/ABs synergism were reported, such as the inhibition of common biochemical pathways, the inhibition of protective enzymes, and the combination of membrane active agents. These mechanisms lead to enhance the diffusion of other antimicrobials [15].

4. CONCLUSION

In conclusion, our study showed the antibacterial effect of *L. cubeba* EO against isolated strains. Lower antibacterial activity (higher MIC values) of *L. cubeba* EO against reference strains (5.53 mg/mL) was found than isolated strains (1.15 - 2.30 mg/mL). Most of combinations showed synergistic effect and the best synergistic effect were obtained for *L. cubeba* and nalidixic acid or oxytetracycline combination against isolate *E. coli* 9C48. The reduction in the effective dose of antimicrobial agents could be useful for treatments in the clinical setting in order to decrease the adverse effects of ABs as well as EOs in the future therapy. The results obtained could be a potential and promising application for sustainable therapy in aquaculture.

Acknowledgments. This work was supported by CIRAD, France and Hanoi University of Sciences and Technology, Viet Nam.

REFERENCES

1. Rodgers C. J., Furones M. D. - Antimicrobial agents in aquaculture: practice, needs and issues, Options Méditerranéennes **86** (2009) 41-59.
2. Raissy M., Khamesipour F., Rahimi E., Khodadoostan A. - Occurrence of *Vibrio* spp., *Aeromonas hydrophila*, *Escherichia coli* and *Campylobacter* spp. in crayfish (*Astacus leptodactylus*) from Iran, Iran J. Fish. Sci. **13** (4) (2014) 944-954.
3. Pham D. K., Chu J., Do N. T., Brose F., Degand G., Delahaut P., De Pauw E., Douny C., Van Nguyen K., Vu T. D., Scippo M.-L., Wertheim H. F. L. - Monitoring antibiotic use and residue in freshwater aquaculture for domestic use in Vietnam, EcoHealth **12** (3) (2015) 480-489.
4. Cabello F. C. - Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment, Environ Microbiol **8** (7) (2006) 1137-1144.
5. Nguyen H. V., Caruso D., Lebrun M., Nguyen N. T., Meile J. C., Chu K. S., Sarter S. - Antibacterial activity of *Litsea cubeba* (Lauraceae, May Chang) and its effects on the biological response of common carp *Cyprinus carpio* challenged with *Aeromonas hydrophila*, J. Appl. Microbiol **121** (2) (2016) 341-351.
6. van Vuuren S., Viljoen A. - Plant-based antimicrobial studies-methods and approaches to study the interaction between natural products, Planta Med. **77** (11) (2011) 1168-1182.

7. Semeniuc C. A., Pop C. R., Rotar A. M. - Antibacterial activity and interactions of plant essential oil combinations against Gram-positive and Gram-negative bacteria, J. Food .Drug. Anal. **25** (2) (2017) 403-408.
8. Gutierrez J., Barry-Ryan C., Bourke P. - Antimicrobial activity of plant essential oils using food model media: Efficacy, synergistic potential and interactions with food components, Food Microbiol **26** (2) (2009) 142-150.
9. Langeveld W. T., Veldhuizen E. J., Burt S. A. - Synergy between essential oil components and antibiotics: a review, Crit. Rev. Microbiol **40** (1) (2014) 76-94.
10. Si H., Hu J., Liu Z., Zeng Z.-l. - Antibacterial effect of oregano essential oil alone and in combination with antibiotics against extended-spectrum β-lactamase-producing *Escherichia coli*, FEMS Immunol Med. Microbiol **53** (2) (2008) 190-194.
11. Saikia A. K., Chetia D., D'Arrigo M., Smeriglio A., Strano T., Ruberto G. - Screening of fruit and leaf essential oils of *Litsea cubeba* Pers. from north-east India – chemical composition and antimicrobial activity, J. Essent Oil. Res. **25** (4) (2013) 330-338.
12. Li W. R., Shi Q. S., Liang Q., Xie X. B., Huang X. M., Chen Y. B. - Antibacterial activity and kinetics of *Litsea cubeba* oil on *Escherichia coli*, PLoS. One. **9** (11) (2014) e110983.
13. Wei L. S., Wee W. - Chemical composition and antimicrobial activity of *Cymbopogon nardus* citronella essential oil against systemic bacteria of aquatic animals, Iran J. Microbiol **5** (2) (2013) 147-152.
14. Mith H., Duré R., Delcenserie V., Zhiri A., Daube G., Clinquart A. - Antimicrobial activities of commercial essential oils and their components against food-borne pathogens and food spoilage bacteria, Food. Sci. Nutr. **2** (4) (2014) 403-416.
15. Yap P. S. X., Lim S. H. E., Hu C. P., Yiap B. C. - Combination of essential oils and antibiotics reduce antibiotic resistance in plasmid-conferred multidrug resistant bacteria, Phytomedicine **20** (8) (2013) 710-713.

TÓM TẮT

NGHIÊN CỨU TƯƠNG TÁC KHÁNG KHUẨN CỦA TINH DẦU MÀNG TANG *LITSEA CUBEBA* VÀ KHÁNG SINH TRONG THUỶ SẢN

Nguyễn Hải Vân[1, 2], Vũ Thu Trang[1], Chu Kỳ Sơn[1], Samira Sarter[2, *]

[1]*Viện Công nghệ sinh học và Công nghệ thực phẩm, Trường Đại học Bách khoa Hà Nội, số 1 Đại Cồ Việt, Hai Bà Trưng, Hà Nội, Việt Nam*

[2]*CIRAD, UMR ISEM, Montpellier F-34398, Pháp*

[*]Email: *samira.sarter@cirad.fr*

Nghiên cứu này đánh giá tương tác kháng khuẩn giữa tinh dầu màng tang (*Litsea cubeba*) và một số kháng sinh đối với 2 chủng vi khuẩn kiểm định và 6 chủng vi khuẩn phân lập từ thuỷ sản bao gồm *Escherichia coli* ATCC 25922 và *Vibrio parahaemolyticus* ATCC 17802; và *E. coli* 9C48, *E. coli* 11C123, *Vibrio* 2S4, *Vibrio* 2N38, *V. parahaemolyticus* ND201 và *V.*

parahaemolyticus TB81. Khả năng kháng khuẩn của tinh dầu, axit nalidixic và oxytetracycline được xác định bằng phương pháp pha loãng liên tục sử dụng phên 96 giếng. Tác dụng kháng khuẩn của tinh dầu màng tang đối với vi khuẩn phân lập (MIC = 1,15 - 2,30 mg/mL) tốt hơn đối với vi khuẩn kiểm định (MIC = 5,53 mg/mL). Hiệu ứng tương tác giữa tinh dầu màng tang và axit nalidixic hoặc oxytetracycline được xác định bằng phương pháp bàn cờ thông qua việc xác định giá trị nồng độ ức chế riêng phần (FIC). Trong 16 tương tác tinh dầu - kháng sinh, có 11 tương tác hiệp đồng (FIC ≤ 0,5), 3 tương tác cộng hợp (0,5 < FIC ≤ 1,0) và 2 tương tác không khác biệt (1,0 < FIC ≤ 4,0). Tương tác hiệp đồng giữa tinh dầu và kháng sinh có thể làm tăng hiệu quả, giảm độc tính, giảm tác dụng phụ, giảm nồng độ sử dụng của kháng sinh được sử dụng trong nuôi trồng thủy sản.

Từ khóa: Litsea cubeba, tinh dầu, hoạt tính kháng khuẩn, tác dụng hiệp đồng.